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Pathogenicity of Aeromonas hydrophila sub species hydrophila causing ulcer in rainbow trout, Oncorhynchus mykiss (Walbaum) in India

Neetu shahi[1], Sumanta K Mallik[1], Monalisa Sahoo[2]

[1]Directorate of Coldwater Fisheries Research, Industrial Area, Bhimtal 263 136, Nainital, Uttarakhand, India.
[2] Indian veterinary Research Institute, Izatnagar, Bareilly 243 122, UtterPradesh
[1]Mobile: + 91 7409148538

E-mail*: shahineetu@rediffmail.com*

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Abstract

From the haemorrhagic and opaque eye of rainbow trout, *Oncorhynchus mykiss* a pure culture of bacteria was isolated. The etiological agent was identified as *Aeromonas hydrophila* subspecies *hydrophila* (strain RTMCX1), which is a highly pathogenic, fast growing bacterium.16S rRNA gene sequences were 99% similar to *A. hydrophila.* This strain, exhibited ß- haemolytic activity against sheep blood and rainbow trout erythrocytes, and showed capability to survive in rainbow trout serum, which may be associated with virulence. RTMCX1survived and grew in presence of 0.5 -10 % NaCl and temperature range of 0.5-35 °C. Mortality was recorded in experimentally infected fish. LD_{50} values of RTMCX1 for intraperitoneal injection were 1.9 x 10^4 cfu/fish. Histology of the injected fish showed rhabdomyolysis in muscle and interstitial nephrititis in kidney with infiltration of mononuclear cells in both the organs. The isolated strain was sensitive to majority of the antibiotics used in aquaculture.

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INTRODUCTION

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), is one of the most valuable species of the coldwater aquaculture industry in India. Culture systems for this species have expanded rapidly since 1950s. In India, intensification of trout farming has resulted in growing problems of mass mortality of fishes, with severe economic loss to trout growers. Most of the time the reasons for sudden death is not known, as majority of the trout farms are located in remote areas, so difficult to reach during several part of the year.

In many countries the trout farming faced the problem of bacterial diseases, such as motile aeromonad septicaemia, vibriosis, fin and tail rot, dropsy, which cause significant economic losses because of mortalities of young and adult rainbow trout (Austin & Austin 2007). However, there is no detailed study or published literature available on bacterial diseases of cultured rainbow trout from India.

The genus *Aeromonas* comprises a collection of gram negative, catalase and oxidase-positive, glucose-fermenting motile rods. It is a ubiquitous inhabitant of aquatic ecosystems, such as freshwater, coastal water and sewage (Monfort & Baleux 1990). These bacteria

have a broad host range, and have often been isolated from humans with diarrhea (Ogunsanya, Rotimi & Adenuga 1994), as well as from fish with 'hemorrhagic septicemia' or 'motile aeromonas septicaemia'. *Aeromonas hydrophila, A. sobria, A. veronii* and *A. salmonicida* are the most important fish pathogens. In fishes, *A. hydrophila* is considered an opportunistic pathogen or secondary invader, but in some conditions such as stress or poor immune function, it may also be a primary pathogen (Thorpe & Roberts 1972). Though there are reports of haemorrhagic septicaemia, ulceration and fin/tail rot caused by *A. hydrophila* to rainbow trout in many countries, no information presently exists regarding pathogenicity of *A. hydrophila* to rainbow trout in Indian coldwater region. The bacterium is a significant contributing factor to poor health and performance of rainbow trout under intensive aquaculture conditions, especially where temperature reach the upper end of the range of tolerance for this species, or when water quality is poor.

In this study, the bacterial isolate from diseased rainbow trout was identified as *A. hydrophila* on the basis of morphological and biochemical characteristics and phylogenetic analysis derived from 16S rRNA sequence. The pathogenicity was confirmed in healthy rainbow trout by intra-peritoneal injection and by the survivability and growth of bacteria in rainbow trout serum.

MATERIALS AND METHODS

Aeromonas hydrophila isolate and reference strains

Sample for bacterial isolation was taken from deep hemorrhagic ulcers of moribund rainbow trout and cultured on Columbia sheep blood agar plates (HiMedia) and Tryptone soya agar (TSA, Difco) at 28.0 °C for 24-96 h. *Aeromonas* Isolation Medium Base (AIMB, HiMedia), supplemented with ampicillin (5.0 μg mL^1), was used to check the colony characteristic of isolated strain. The isolate was subjected to biochemical and molecular identification and also analysed by standard microscopic techniques. These isolates were maintained frozen at -80.0 °C in bacteria freezing media tubes (AMRESCO) until further use. The reference strains used in this study were *A. hydrophila* ATCC 35654 and *A. hydrophila* ATCC 7965.

Physiological and biochemical characteristics

The following tests were carried out: Gram stain, motility and cell morphology (phase-contrast microscopy after growth in TSA broth for 24 h); susceptibility to 10 and 150 μg of vibriostatic agent O/129; growth in 1 % peptone at 4.0, 10.0, 35.0 and 40.0 °C (7 days); growth in 1% peptone at 0.5, 2.0, 6.0, 8.0 and 10 % salt concentrations (7 days), cytochrome-oxidase, O/F test; production of H_2S on triple sugar iron agar (Difco TM), ONPG (ß-galactosidase, Hi-media), indole and Voges-Proskauer (VP) tests; gas production from glucose, nitrate-reduction, acid production from sugar. The following enzymatic activities were also determined: catalase (3 % H_2O_2), urease, gelatinase, starch hydrolysis, haemolysis of sheep blood (sheep blood agar plates with 5 % blood) and rainbow trout erythrocytes (5 % in blood agar base).

Antibiotic susceptibility pattern

The antibiotic susceptibility pattern of the isolated bacteria was determined using the standardized disc diffusion method on Mueller-Hinton agar (Difco). The following chemotherapeutic reagents (HiMedia, μg per disc) were used: Ampicillin (AMP ^{10} μg), Chloroamphenicol (C ^{10} μg), Penicillin (P ^{2} μg), Erythromycin (E ^{10} μg), Tetracyclin (TE ^{10} μg), Nalidixic acid (NA ^{30} μg), Rifampicin (RIF ^{30} μg), Cloxacilin (COX^{30} μg), Clindomycin (CD ^{10} μg), Ofloxacin (OF ^{5} μg), Vancomycin (VA ^{10} μg), Azithromycin (AZM ^{30} μg), Amoxycillin (AMX ^{10} μg), Gentamicin (GEN ^{30} μg), Neomycin (N ^{30} μg), Streptomycin (S ^{25} μg), Vancomycin (VA ^{10} μg), Cefalexin (CN ^{30} μg), Cefotaxime (CTX ^{10} μg), Bacitracin (B ^{10} μg), Co-Trimoxazole (COT ^{25} μg), Cefaclor (CF ^{30} μg), Ciprofloxacin (CIP ^{30} μg), Kanamycin (K 30 μg), Methicillin (MET ^{10} μg), Novobiocin (NV ^{30} μg), Sulphadiazine (SZ ^{100} μg), Trimethoprim (TR ^{30} μg). The diameter of each zone of inhibition was determined after 24-30 h of incubation at 28 °C, and the antibiograms interpreted in agreement with the National Committee for Clinical Laboratory Standards recommendations. Reference strains *A. hydrophila* ATCC 35654 and *A. hydrophila* ATCC 7965 were also used for comparison.

16S rRNA gene sequence analysis

Chromosomal DNA from pure cultures was extracted using high pure PCR template preparation kit for genomic DNA (Roche Version 16.0) according to the manufacturer's instructions. The concentration of each DNA sample was checked using Qubit 2.0 instrument (Invitrogen), and adjusted to 20-25 ng μL^{-1}. PCR amplification of the 16S rRNA gene of the isolates was performed using the universal primers. The 16S rRNA gene sequence was determined directly using the PCR-amplified DNA as a sequencing template. The re-

sulting sequences were compared to those available in GenBank, EMBL (http://www.ebi.ac.uk) and the National Centre for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) program. The sequence of the 16S rRNA were aligned with sequences from other *Aeromonas* species and related organisms using the CLUSTAL X 2.0 program (Thompson, Gibson, Plewniak, Jeanmougin & Higgins 1997), and a phylogenetic tree was constructed using the neighbor-joining method by the MEGA 4.1 software (Kumar, Tamura & Nei 2004). The accuracy of the resulting tree was measured by bootstrap resampling of 1000 replicates. The nucleotide sequences for 16S rRNA generated in this study were deposited in the NCBI GenBank database under the accession number JX390650.

Survival ability in rainbow trout serum

Bacteria grown in nutrient broth at 28 °C for overnight were collected by centrifugation and then washed three times in FTA hemagglutination buffer (BD, USA), pH 7.2. The bacterial pellets were resuspended with fresh rainbow trout serum and adjusted to 5.0 x10^6 colony-forming units (CFU) mL^{-1} of bacterial cells. One hundred microlitres of each sample was removed at 0, 1, 3, 6, 8, 12 and 24 h after incubation at 28 °C, and serial tenfold dilution in FTA hemagglutination buffer were spread on TSA. Total plate count was taken 24-30 h of incubation at 28 °C

Pathogenicity test in rainbow trout

The in vivo pathogenicity of *A. hydrophila* in rainbow trout was evaluated using the median lethal dose (LD_{50}). The LD_{50} value was determined by the method of Reed & Muench (1938) using decreasing doses from 10^8 to 10^2 CFU mL^{-1}, with 20 fish per treatment group (average weight of fish 17.0 ± 2.5 g). Fishes were injected intra-peritoneal with 0.1 mL bacterial suspension per individual, and mortality was monitored for 2 weeks, until no dead fish were observed. The control group was injected with an equal volume of sterile phosphate buffer saline. Injected and control fish were analysed by histopathological and microbiological methods. Mortality was attributed to the inoculated bacterium if the injected organism was recovered in pure culture from the internal organs (liver, anterior kidney, posterior kidney, gall bladder and spleen). Tissue samples were fixed in 10 % neutral buffered formaldehyde followed by processing for paraffin-wax embedding. Sections were cut at 4 μm thickness and stained with haematoxylin and eosin (H&E). The stained sections were examined under a light microscope (Leica, DMLS, TK-C1380E).

RESULTS

Morphological, physiological and biochemical characteristics

A. hydrophila isolate RTMCX1 grew well on NA, TSA and AIMB, after incubation at 28 °C for 24 - 48 h. Colonies were circular, humid, smooth and creamy color after 48 h incubation at 28 °C on NA and TSA, and dark green, opaque with dark centre on AIMB. The isolate RTMCX1 was short rod-shaped, occurring in singly or in pairs or as short chains with a diameter of 0.4-1.5 μm. RTMCX1 was gram negative, catalase positive, motile, fermentative and resistant to the vibriostatic compound O/129. RTMCX1 and reference strain could grow in 0.5 %, 2.0 %, 4.0 % and 6.0 %, 8.0 % and 10.0 % NaCl. RTMCX1 was positive for nitrate reduction, VP, hydrolysis of gelatin and esculin, arginine dihydrolysis and negative for the production of H_2S, methy red, indole production and dihydrolysis of lysine and ornithine. All (RTMCX1 and reference strain) produced acid from dextrose, galactose, mannose, maltose, sucrose and trehalose. ß hemolysin was positive for sheep and rainbow trout erythrocytes. Some variability was observed in the utilization of sugars.

Antimicrobial susceptibility result

The RTMCX1 was sensitive to majority of the antibiotics tested. Some variations of susceptibility between strains were observed, but all were resistant to ampicillin (AMP ^{10} μg), cefalexin (CN ^{30} μg), cloxacillin (COX ^{30} μg), amoxicillin (AMX ^{10} μg) and penicillin (P ^{2} μg).

Phylogenetic analysis based on 16SrRNA genes

Sequencing analysis of the PCR amplified 1400 bp 16S rRNA gene revealed that the rainbow trout isolate was 99% dentical with *A. hydrophila* subsp. *hydrophila* strain. In a phylogenetic tree based on the neighbor-joining algorithm, *A. hydrophila* isolate RTMCX1 clustered with *A. hydrophila* JF 713703 and *A. hydrophila* subsp. *hydrophila* AB 680394 with 16S rRNA gene sequence similarity levels of 99 % and bootstrap value >90 % (Fig 1). The RTMCX 1 has an additional base G at position 20 of the sequence, whereas all the compared strains do not have an additional base at that position (Fig. 2).

Survival ability in rainbow trout serum

The cell numbers of the isolated strains decreased during the first 3 h of incubation. The strain subsequently recovered and increased in cell number However, ref-

erence strain *A. hydrophila* ATCC 35654 cell number decreased during the incubation period and could not recover.

Pathogenicity of isolate

For rainbow trout, cumulative mortality rates were 80 % at day 2 and 100 % at day 3 , post infection at high doses (4.9 x 10^8 CFU mL^{-1}). When the concentration was below 3.9 x10^2 CFU mL^{-1}, no deaths were observed for 5 days. RTMCX1 was highly pathogenic to rainbow trout, with 100 % mortality within 12-13 days of i.p injection, at LD_{50} value of 1.9 x 10^4 CFU g^{-1} body weight. The control fishes, which were injected with PBS, did not suffer any mortality after 13 – 14 days post injection. Most of the dead fish challenged by intra-peritoneal injection developed cutaneous ulcers and reddening at injection sites. Pure culture of *A. hydrophila* was re-isolated and identified from the kidney and gall bladder of the moribund fish, and found to be same as that of the injected strain. Darkening of kidney and swelling of gall bladder was observed in all the dead fishes, while none of the control fish exhibited any clinical sign of disease.

HISTOPATHOLOGY

Severe changes were observed in the kidney muscle and gall bladder of injected fishes (Fig. 3-Fig. 5). Rhabdomyolysis in the injected site of muscle and interstitial nephrititis in kidney was observed. Histopathological examination of infected fish revealed the marked infiltration of mononuclear cells in kidney and gall bladder of fishes. Disintegration of kidney tubules was also noted. In other organs no marked changes was observed.

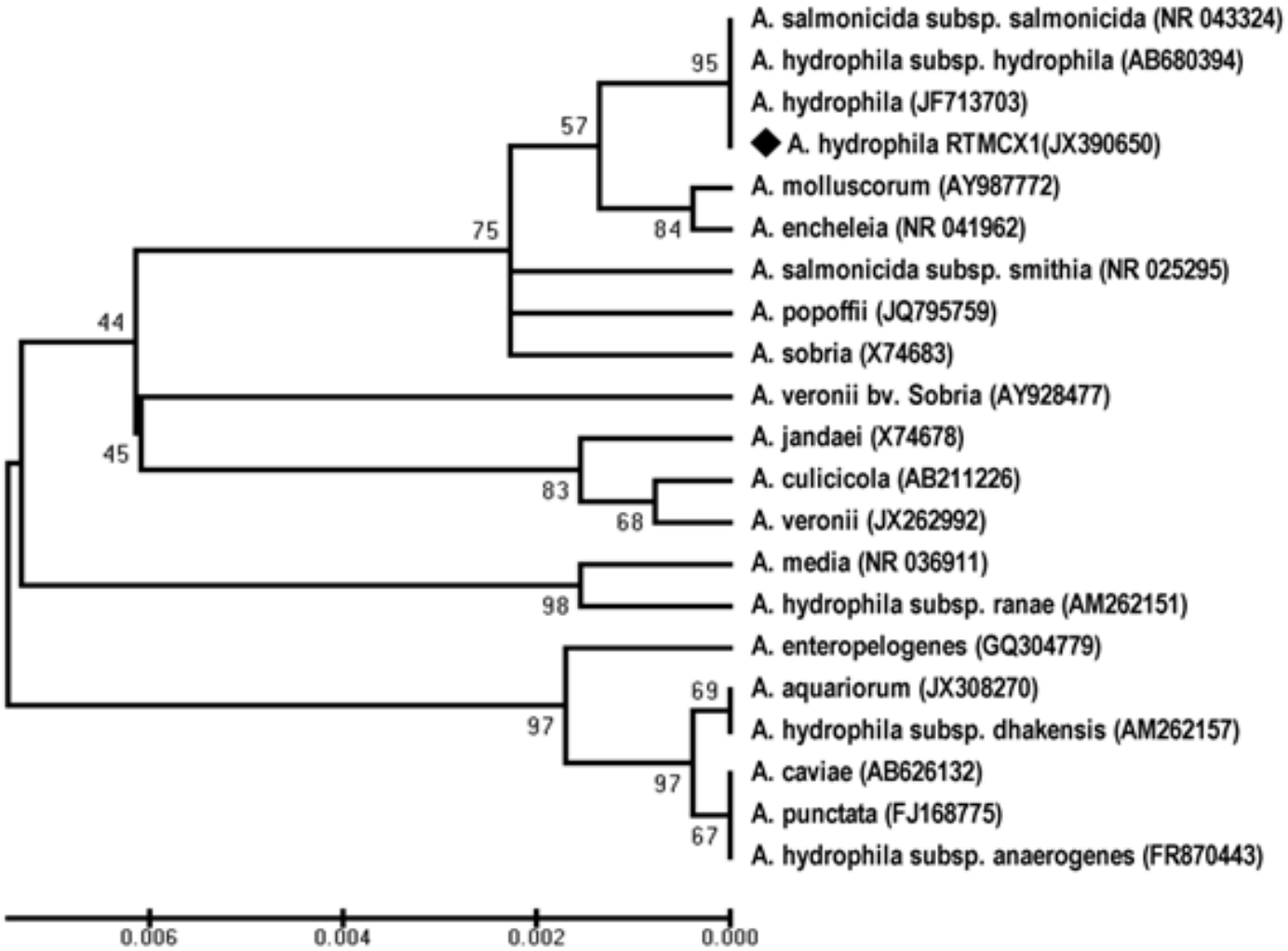

Figure 1: Phylogenetic tree of *Aeromonas hydrophila* isolate RTMCX1 based on 16S rRNA gene sequence. The tree was constructed with the neighbor joining method. Bootstrap values (expressed as percentage of 1000 replicates) are shown at branch points. GeneBank accession numbers are given in parentheses.

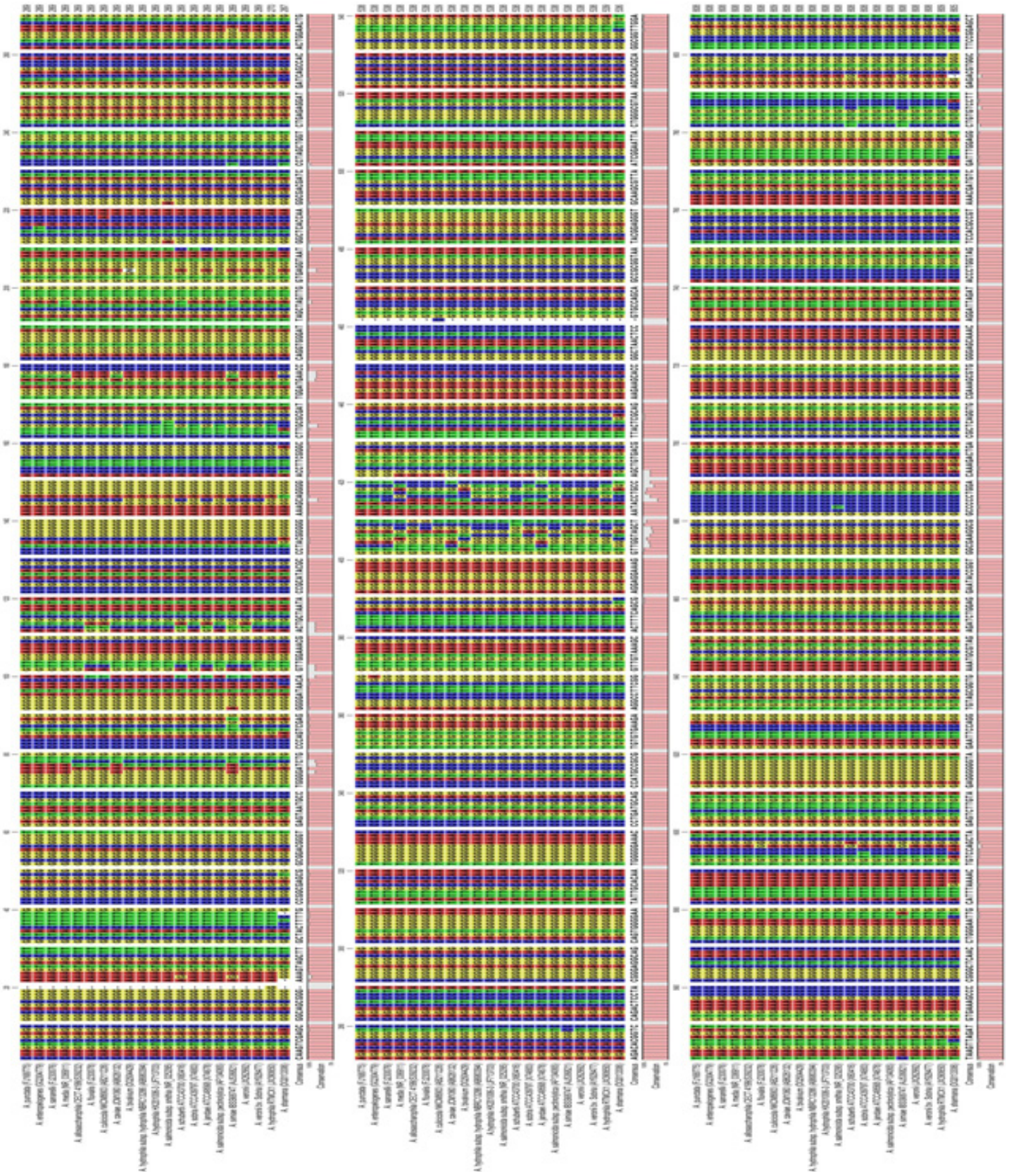

Figure 2: Sequence alingement of RTMCX1 and reference strains

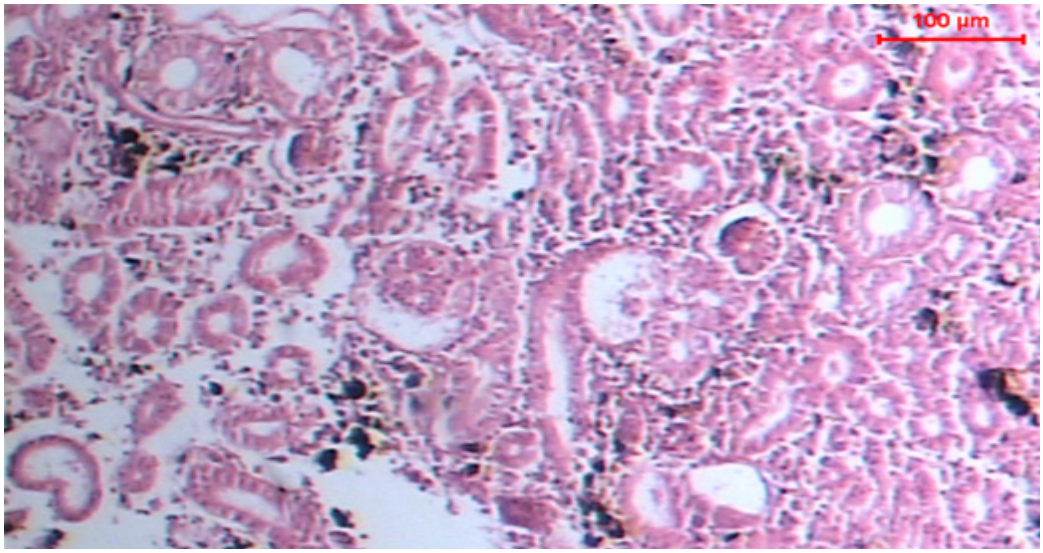

Figure 3: Renal tubules showing extensive degenerative changes with infiltration of inflammatory cells

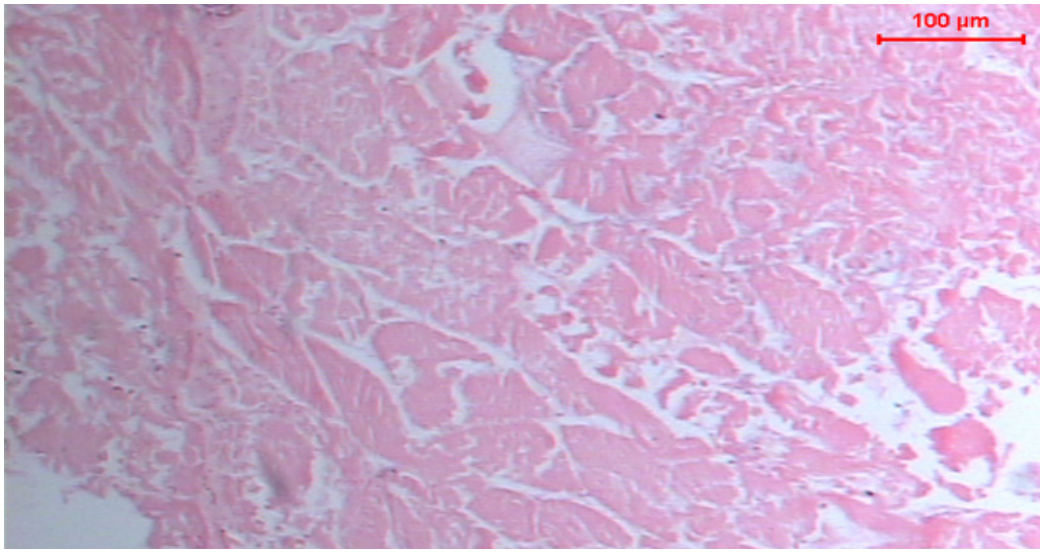

Figure 4: Muscle fibre showing extensive degenerative changes with infiltration of inflammatory cells

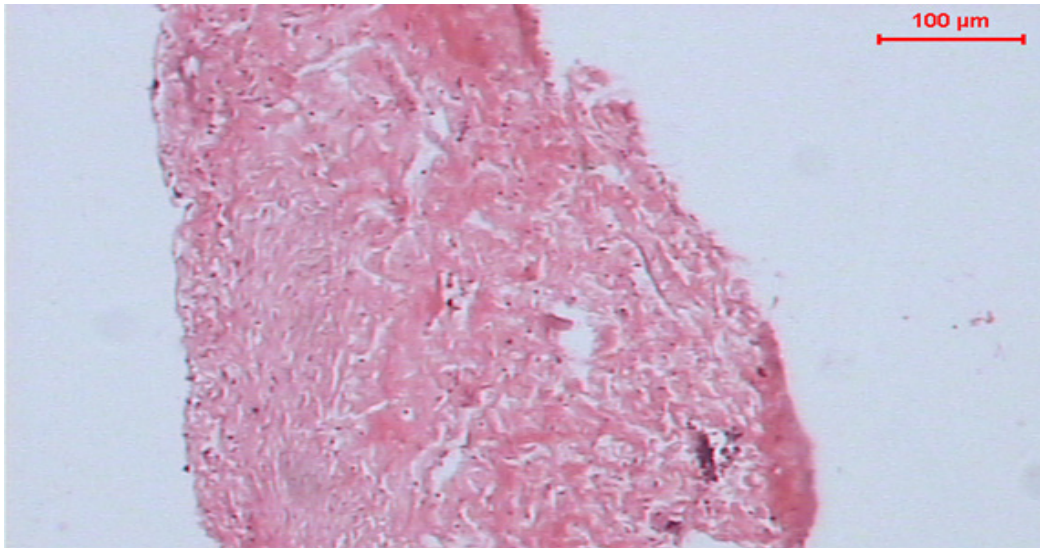

Figure 5: Gall bladder showing mild infiltration of inflammatory cells

REFERENCES

1. Austin B. & Austin D. A. **2007** Bacterial Fish Pathogens, Disease of Farmed and Wild Fish, 4th edn. Springer Praxis, Godalming.
2. Monfort P. & Baleux B. **1990** Dynamics of *Aeromonas hydrophila, Aeromonas sobria,* and *Aeromonas caviae* in a sewage treatment pond. *Applied Environmental Microbiology* 56, 1999-2006.
3. Ogunsanya T. I., Rotimi V. O. & Adenuga A. **1994** A study of the aetiological agents of childhood diarrhea in Lagos, Nigeria. *Journal of Medical Microbiology* 40, 10-14.
4. Thorpe J. E. & Roberts R. J. **1972** Aeromonad epidemic in brown trout (Salmo trutta L.). *Journal of Fish Biology 4, 441-451.*
5. Thompson J. D., Gibson T. J., Plewniak F, Jeanmougin F. & Higgins D. G. **1997** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Resesearch* 25, 4876-4882.
6. Kumar S., Tamura K. & Nei M. **2004** MEGA 4.1: integrated software for molecular evolutionary genetic analysis and sequence alignment. *Briefing in Bioinformatics* 5, 150-163.
7. Reed L. J. & Muench H. **1938** A simple method of estimating fifty percent endpoints. *American Journal of Hygiene* 27, 493-497.

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